

**This Page Is Inserted by IFW Operations
and is not a part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/53, 33/48, 33/543	A1	(11) International Publication Number: WO 99/40434 (43) International Publication Date: 12 August 1999 (12.08.99)
(21) International Application Number: PCT/US99/02442 (22) International Filing Date: 4 February 1999 (04.02.99) (30) Priority Data: 60/073,605 4 February 1998 (04.02.98) US (71) Applicant: INVITROGEN [US/US]; 1600 Paraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors: HOFFFLER, James, P.; 957 Jasmine Court, Carls- bad, CA 92009 (US). FERNANDEZ, Joseph, M.; 3930 Skyline, Carlsbad, CA 92008 (US). NASOFF, Marc, S.; 11734 Mira Lago Way, San Diego, CA 92131 (US). (74) Agent: REITER, Stephen, E.; Gray Cary Ware & Freidenrich LLP, Suite 1600, 4365 Executive Drive, San Diego, CA 92121 (US).	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	
(54) Title: MICROARRAYS AND USES THEREFOR (57) Abstract The invention disclosed herein comprises methods of using microarrays to simplify analysis and characterization of genes and their function. In one aspect of the invention the methods are used to identify and characterize antibodies having binding affinity for a specific target antigen. The invention further comprises a method of determining gene expression at the protein level comprising contacting an array of characterized or uncharacterized antibodies on a solid surface with one or more proteins and identifying the antibodies to which said protein(s) binds. This method can be additionally used to compare the protein expression in two different populations of cells, such as normal cells and cancer cells or resting cells and stimulated cells. A further aspect of the invention comprises a method of determining gene expression at the protein level comprising contacting a microarray of nucleic acid samples derived from a variety of different sources with one or more nucleic acid probes then identifying the sample or samples to which the probe binds.		

MICROARRAYS AND USES THEREFORField of the Invention

The invention disclosed herein relates to new
5 methods of using microarray technologies. The methods
are useful for identifying and characterizing specific
antibodies as well as the characterization of different
tissues or cells by protein or nucleic acid analysis.

10

Background of the Invention

Recent breakthroughs in nucleic acid sequencing
technology have made possible the sequencing of entire
genomes from a variety of organisms, including humans.
15 The potential benefits of a complete genome sequence are
many, ranging from applications in medicine to a greater
understanding of evolutionary processes. These benefits
cannot be fully realized, however, without an
understanding of how and where these newly sequenced
20 genes function.

Traditionally, functional understanding started with
recognizing an activity, isolating a protein associated
with that activity, then isolating the gene, or genes,
encoding that protein. The isolated protein was also
25 used to generate antibody reagents. Specific antibodies

and fragments of the isolated gene were both employed to study tissue expression and function.

Several methods have been used to study protein expression patterns including *in situ* hybridization studies of tissue sections and Northern blots. These methods are both time consuming and require relatively large amounts of material to perform successfully.

Antibodies that bind to specific antigens have been produced by a variety of methods including immunization of animals, fusion of mammalian spleen cells to immortalized cells to produce hybridomas, random peptide generation using phage or bacterial display and constrained peptide libraries. Regardless of how the desired antibody is generated, the methods currently available to identify one with a particular binding specificity are generally laborious and incapable of the simultaneous testing of large numbers of unknowns.

One method involves binding the antigen to a porous membrane, such as nitrocellulose, contacting the membrane with a source of test antibodies, then determining whether or not any of the test antibodies has bound to the antigen. This method only allows the testing of one source of test antibodies per piece of porous membrane, making the method both inconvenient and wasteful of materials.

Antibody/antigen reactions can also be evaluated in plastic plates, such as 96-well microtiter plates, using methods similar to those described above. This method is likewise limited in the number of samples that can be tested in any one assay, thus requiring many assays to fully evaluate a large number of antibody unknowns. Chang (US Patent No. 4,591,570, issued 5/27/86) describes an array of a limited number of characterized antibodies to known antigens on a glass surface that can be used to bind to specific antigens on the surface of whole cells.

Recently new technologies have arisen that allow the creation of microarrays containing thousands or millions of different elements. Such array technology has been applied mainly to forming arrays of individual nucleic acids (see, for example, Marshall and Hodgson, *Nature Biotech.* 16:27-31, 1998; Ramsay, *Nature Biotech.* 16:40-44, 1998), in particular short oligonucleotides synthesized *in situ*.

Methods are needed to simply and rapidly screen very large numbers of uncharacterized antibodies for those specific for a given antigen as well as for the characterization of tissues and cells by nucleic acid and/or protein analysis. The invention described herein addresses that need.

Brief Description of the Invention

The invention disclosed herein comprises methods of using microarrays to simplify analysis and characterization of genes and their function. In one aspect of the invention the methods are used to identify and characterize antibodies having binding affinity for a specific target antigen. This method comprises contacting an array of uncharacterized antibodies bound to a solid surface with at least one target antigen and identifying the antibodies to which the target antigen binds. The method can be performed under a variety of conditions to identify antibodies with a range of binding affinities.

A second aspect of the invention comprises a method of determining gene expression at the protein level comprising contacting an array of characterized or uncharacterized antibodies on a solid surface with one or more proteins and identifying the antibodies to which said protein(s) binds. This method can be further used to compare the protein expression in two different populations of cells, such as normal cells and cancer cells or resting cells and stimulated cells. A related embodiment can be used as a tool in the diagnosis of various disorders.

A further aspect of the invention comprises a method of determining gene expression at the protein level comprising contacting a microarray of nucleic acid samples derived from a variety of different sources with

one or more nucleic acid probes then identifying the sample or samples to which the probe binds.

Brief Description of the Figures

5 Figures 1A, 1B, and 1C show microarrays of antibodies bound to positively charged nylon, reacted with antigen and detected by non-fluorescent means.

Figure 2 shows a microarray produced using a robotic arraying apparatus. Antigen binding is detected by non-
10 fluorescent means.

Figure 3 shows the ability of the antibody microarrays to evaluate relative binding affinities to a specific antigen.

Figure 4 shows a microarray of polyclonal antibodies
15 in comparison to a microarray of monoclonal antibodies.

Figures 5A, 5B and 5C show a microarray of antibodies reacted with a cell lysate under conditions that vary the amount of background binding.

Detailed Description of the Invention

20 The present invention discloses methods of using microarrays to simplify analysis and characterization of genes and their function. In a first aspect of the invention the methods are used for identifying and characterizing antibodies having binding specificity to a

particular antigen or set of antigens. This method utilizes microarray technology to create ordered matrices of large numbers of uncharacterized antibodies which can then be contacted with antigen under a variety of conditions. The method is rapid and simple to perform and is applicable to the simultaneous screening of very large numbers of antibodies.

Briefly, uncharacterized antibodies are bound to a solid surface in an array format consisting of discrete spots whose spatial location can be easily identified. Each location represents an antibody from a known source, such as a particular hybridoma growing in a well in a 96-well microtiter plate. The space between the antibody spots is treated to minimize non-specific binding to the solid support. The arrayed antibodies are then contacted with an antigen, or a set of antigens, for which specific antibodies are sought. The antigen solution is left in contact with the array for an amount of time sufficient to allow antigen:antibody complexes to form (generally 10 minutes to 2 hours), then the unbound antigen is washed away under suitable conditions. Bound antigen is detected at a particular antibody spot using one of a variety of detection methods, thus identifying the source of an antibody specific for the particular antigen.

The term "antibody" is used herein in the broadest sense and specifically includes intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments,

WO 99/40434

PCT/US99/02442

7

including single chain antibodies, so long as they exhibit the desired binding properties as described herein.

Various procedures well-known in the art may be used
5 for the production of polyclonal antibodies to an epitope or antigen of interest. A host animal of any of a number of species, such as rabbits, goats, sheep, horse, cow, mice, rats, etc. is immunized by injection with an antigenic preparation which may be derived from cells or
10 microorganisms, or may be recombinantly or synthetically produced. Various adjuvants well known in the art may be used to enhance the production of antibodies by the immunized host, for example, Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide,
15 surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, liposomes, potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Propionibacterium acnes*, and the like.

20 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring
25 mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies

The monoclonal antibodies contemplated for use
25 herein specifically include "chimeric" antibodies
(immunoglobulins) in which a portion of the heavy and/or
light chain is identical with or homologous to
corresponding sequences in antibodies derived from a
particular species or belonging to a particular antibody

class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as
5 fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine)
10 antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized
15 antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity,
20 affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in
25 the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or
30 substantially all of the CDR regions correspond to those

WO 99/40434

11

PCT/US99/02442

Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

Large quantities of single chain antibodies with uncharacterized randomized binding specificity can be produced using a number of methodologies known in the art. Recombinant antibody libraries can be created in filamentous phage particles (Daniels and Lane, *Methods* 9(3):494-507, 1996; Reichmann and Weill, *Biochemistry* 32(34):8848-8855; Rader and Barbas, *Curr Opin Biotechnol* 9(4):503-508, 1997; Iba and Kurosawa, *Immunol Cell Biol* 75(2):217-221, 1997, WO 90/05144, WO 92/01047, WO 92/20791, WO 93/19172, GB 9722131.8, GB9810228.8 and GB 9810223.9, all of which are incorporated by reference herein in their entirety), for example, or similarly in yeast, bacteria, and the like. Other methods for creating random libraries of sFvs include various solid state synthesis methods.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain ($V_H - V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

The antibodies employed in the invention can be isolated prior to creating a microarray. An "isolated" molecule, whether an antibody, antigen or nucleic acid, is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with particular uses for the molecule, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, a protein will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes the protein *in situ* within recombinant cells since at least one component of the protein's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step. Unpurified antibodies, such as those found in serum, can also be employed in the present invention.

By "isolated" in reference to nucleic acid is meant a polymer of 14, 17, 21 or more contiguous nucleotides, including DNA or RNA that is isolated from a natural source or that is synthesized. The isolated nucleic acid of the present invention is unique in the sense that it is not found in a pure or separated state in nature. Use

of the term "isolated" indicates that a naturally occurring sequence has been removed from its normal cellular (i.e., chromosomal) environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide sequence present, but that it is essentially free (about 90 - 95% pure at least) of non-nucleotide material naturally associated with it and thus is meant to be distinguished from isolated chromosomes.

One particularly useful method of isolating antibodies, such as single chain antibodies from a cell extract, is affinity purification. Resins suitable for antibody purification are well known in the art, for example, protein A sepharose. A recombinant antibody can be engineered to contain an affinity purification tag to facilitate its purification. Resins suitable for antibody purification are well known in the art, for example, protein A sepharose.

Affinity purification tags are generally peptide sequences that can interact with a binding partner immobilized on a solid support. Synthetic DNA sequences encoding multiple consecutive single amino acids, such as histidine, when fused to the expressed protein, may be used for one-step purification of the recombinant protein by high affinity binding to a resin column, such as nickel sepharose. An endopeptidase recognition sequence can be engineered between the polyamino acid tag and the protein of interest to allow subsequent removal of the

leader peptide by digestion with enterokinase, and other proteases. Sequences encoding peptides such as the chitin binding domain (which binds to chitin), biotin (which binds to avidin and strepavidin), and the like can
5 also be used for facilitating purification of the protein of interest. The affinity purification tag can be separated from the protein of interest by methods well known in the art, including the use of inteins (protein self-splicing elements, Chong, et al, Gene 192:271-281,
10 1997).

By using an amount of resin with binding sites sufficient for only a small portion of the antibody present in the unpurified mixture, the process of isolation can be used to simultaneously normalize yield
15 and isolate the antibody. For example, although each sample will contain a different and unknown amount of antibody protein, the samples can be contacted with an amount of resin whose maximum binding capacity is 10 mgs. Thus any antibody greater than this amount will pass
20 through the resin unbound. The maximum bound amount can then be eluted from the resin.

Methods for creating microarrays are known in the art including printing on a solid surface using pins (passive pins, quill pins, and the like) or spotting with
25 individual drops of solution. Passive pins draw up enough sample to dispense a single spot. Quill pins draw up enough liquid to dispense multiple spots. Bubble printers use a loop to capture a small volume which is dispensed by pushing a rod through the loop.

Microdispensing uses a syringe mechanism to deliver multiple spots of a fixed volume. In addition, solid supports, can be arrayed using pizoelectric (ink jet) technology, which actively transfers samples to a solid support.

One method is described in Shalon and Brown (WO 95/35505, published 12/28/95) which is incorporated herein by reference in its entirety. The method and apparatus described in Shalon and Brown can create an array of up to six hundred spots per square centimeter on a glass slide using a volume of 0.01 to 100 nl per spot. Suitable concentrations of antibody range from about 1 ng/ μ l to about 1 μ g/ μ l. In the present invention, each spot can contain one or more than one distinct antibody.

Other methods of creating arrays are known in the art, including photolithographic printing (Pease, et al, PNAS 91(11):5022-5026, 1994) and *in situ* synthesis. While known *in situ* synthesis methods are less useful for synthesizing polypeptides long enough to be antibodies, they can be used to make polypeptides up to 50 amino acids in length, which can serve as binding proteins as described below.

The microarrays can be created on a variety of solid surfaces such as plastics (eg. polycarbonate), complex carbohydrates (eg. agarose and sepharose), acrylic resins (eg. polyacrylamide and latex beads), and nitrocellulose. Preferred solid support materials include glass slides, silicon wafers, and positively charged nylon. Specific

examples of suitable solid supports are described in the Examples below.

Methods for covalent attachment of antibodies to a solid support are known in the art. Examples of such methods are found in Bhatia, et al, Anal. Biochem. 178(2):408-413, 1989; Ahluwalia, et al, Biosens. Bioelectron. 7(3):207-214, 1992; Jonsson, et al, Biochem. J. 227(2):373-378, 1985; and Freij-Larsson, et al, Biomaterials 17(22):2199-2207, 1996, all of which are incorporated by reference herein in their entirety. Proteins may additionally be attached to a solid support using methods described in the examples below.

Methods of reducing non-specific binding to a solid surface are well known in the art and include washing the arrayed solid surface with bovine serum albumin (BSA), reconstituted non-fat milk, salmon sperm DNA, porcine heparin, and the like (see Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995).

The arrays used to identify antigen-specific antibodies are contacted with a solution containing one or more known antigens in order to identify antibodies in the array with binding specificity for the antigen. The antigens are often proteins, although they may also be organic chemical compounds, carbohydrates, nucleic acids, and the like. They may be isolated or semi-isolated, recombinant or naturally occurring. The amount of antigen used can vary from about 1 - 100 ng/ μ l. The antigen is left in contact with the array for an amount of time sufficient for antibody:antigen complexes to

form, should one of the antibodies in the array be specific for the antigen. The amount of time sufficient for this purpose will range from 5 minutes to 24 hours, and will generally be from 0.5 to 2 hours.

5 One antigen of particular interest in the practice of the invention is recombinant protein, either a full-length gene product or a fragment thereof, for example an Expressed Sequence Tag (or EST fragment). EST fragments are relatively short cDNA sequences that have been
10 randomly generated and sequenced, generally as part of an ongoing effort to map an entire genome (Adams, et al, Science 252(5013):1651-1656, 1991). Large numbers of these sequences are available in public databases. The identity of the proteins encoded by the vast majority of
15 these sequences is unknown. The following discussion, although directed to the expression of EST-encoded peptides, is equally applicable to any expressed product of a nucleic acid sequence, including full-length proteins.

20 Techniques are available in the art by which cells can be genetically engineered to express the peptide encoded by a given EST fragment. The methods of the invention can then be used to identify antibodies specific for the peptide. These antibodies are then
25 useful as reagents that can be employed in purification and identification of the full-length protein, and in other experimental procedures designed to elucidate the protein's location and function.

Prokaryotic hosts are, generally, very efficient and convenient for the production of recombinant proteins and are, therefore, one type of preferred expression system for EST fragments. Prokaryotes most frequently are
5 represented by various strains of *E. coli*. However, other microbial strains may also be used, including other bacterial strains.

In prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a
10 species compatible with the host may be used. Examples of suitable plasmid vectors may include pBR322, pUC118, pUC119, and the like; suitable phage or bacteriophage vectors may include λ gt10, λ gt11, and the like; and suitable virus vectors may include pMAM-neo, PKRC and the
15 like. Preferably, the selected vector of the present invention has the capacity to replicate in the selected host cell.

Recognized prokaryotic hosts include bacteria such as *E. coli* and those from genera such as *Bacillus*,
20 *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, and the like. However, under such conditions, the polypeptide will not be glycosylated. The prokaryotic host selected for use herein must be compatible with the replicon and control sequences in the expression plasmid.

25 To express an EST fragment in a prokaryotic cell, it is necessary to operably link the gene sequence to a functional prokaryotic promoter such as the T7 promoter or RSC promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e.,

inducible or derepressible). Examples of constitutive promoters include the int promoter of bacteriophage λ , the bla promoter of the β -lactamase gene sequence of pBR322, the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage (P_L and P_R), the trp, reca, lacZ, LacI, and gal promoters of *E. coli*, the α -amylase (Ulmanen *et al.*, *J. Bacteriol.* 162:176-182, 1985) and the sigma-28-specific promoters of *B. subtilis* (Gilman *et al.*, *Gene* 32:11-20(1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (1982)), *Streptomyces* promoters (Ward *et al.*, *Mol. Gen. Genet.* 203:468-478, 1986), and the like. Exemplary prokaryotic promoters are reviewed by Glick (*J. Ind. Microbiol.* 1:277-282, 1987); Cenatiempo (*Biochimie* 68:505-516, 1986); and Gottesman (*Ann. Rev. Genet.* 18:415-442, 1984).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene sequence-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold *et al.* (*Ann. Rev. Microbiol.* 35:365-404, 1981). The selection of control sequences, expression vectors, transformation methods, and the like, are dependent on the type of host cell used to express the gene.

Host cells which may be used in the expression systems of the present invention are not strictly

limited, provided that they are suitable for use in the expression of the peptide of interest. Suitable hosts may often include eukaryotic cells. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, 5 and mammalian cells either in vivo, or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO, 3T3 or CHOK1, HEK 293 cells or cells of lymphoid origin (such as 32D cells) and their derivatives. Preferred mammalian 10 host cells include SP2/0 and JS58L, as well as neuroblastoma cell lines such as IMR 332 and PC12 which may provide better capacities for correct post-translational processing.

In addition, plant cells are also available as 15 hosts, and control sequences compatible with plant cells are available, such as the cauliflower mosaic virus 35S and 19S, nopaline synthase promoter and polyadenylation signal sequences, and the like. Another preferred host is an insect cell, for example the *Drosophila* larvae. 20 Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used: Rubin, Science 240:1453-1459, 1988). Alternatively, baculovirus vectors can be engineered to express large amounts of peptide encoded by an EST fragment in insects cells (Jasny, 25 Science 238:1653, 1987); Miller et al., In: Genetic Engineering (1986), Setlow, J.K., et al., eds., Plenum, Vol. 8, pp. 277-297).

Any of a series of yeast gene sequence expression systems can be utilized which incorporate promoter and

termination elements from the actively expressed gene sequences coding for glycolytic enzymes which are produced in large quantities when yeast are grown in media rich in glucose. Known glycolytic gene sequences
5 can also provide very efficient transcriptional control signals. Yeast provides substantial advantages in that it can also carry out posttranslational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high
10 copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene sequence products and secretes peptides bearing leader sequences (i.e., pre-peptides). For a mammalian host,
15 several possible vector systems are available for the expression of and EST fragment.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and
20 translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression.
25 Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the gene sequences can
30 be modulated. Of interest are regulatory signals which

are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Expression of an EST fragment in eukaryotic hosts involves the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the promoter of the mouse metallothionein I gene sequence (Hamer et al., J. Mol. Appl. Gen. 1:273-288, 1982); the TK promoter of Herpes virus (McKnight, Cell 31:355-365, 1982); the SV40 early promoter (Benoist et al., Nature (London) 290:304-310, 1981); the yeast gal4 gene sequence promoter (Johnston et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975, 1982); Silver et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955, 1984), the CMV promoter, the EF-1 promoter, and the like.

An EST fragment and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule (a plasmid). Since such molecules are incapable of autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent or stable expression may occur through the integration of the introduced DNA sequence into the host chromosome.

A vector may be employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by
5 also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The
10 selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by cotransfection. Common selectable marker gene sequences include those for resistance to antibiotics such as ampicillin,
15 tetracycline, kanamycin, bleomycin, streptomycin, hygromycin, neomycin, Zeocin™, and the like. Selectable auxotrophic gene sequences include, for example, hisD, which allows growth in histidine free media in the presence of histidinol.

20 Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such
25 elements include those described by Okayama, Mol. Cell. Bio. 3:280, 1983.

The recombinant antigen may be produced as a fusion protein. When two protein-coding sequences not normally associated with each other in nature are in the same

reading frame the resulting expressed protein is called a "fusion protein" as two distinct proteins have been "fused" together. Fusion proteins have a wide variety of uses. For example, two functional enzymes can be fused
5 to produce a single protein with multiple enzymatic activities or short peptide sequences, such as epitope tags or affinity purification tags (see above), can be fused to a larger protein and serve as aids in purification or as means of identifying the expressed
10 protein by serving as epitopes detectable by specific antibodies.

Epitope tags are short peptide sequences that are recognized by epitope-specific antibodies. A fusion protein comprising a recombinant protein and an epitope
15 tag can be simply and easily purified using an antibody bound to a chromatography resin. The presence of the epitope tag furthermore allows the recombinant protein to be detected in subsequent assays, such as Western blots, without having to produce an antibody specific for the
20 recombinant protein itself. Examples of commonly used epitope tags include V5, glutathione-S-transferase (GST), hemagglutinin (HA), the peptide Phe-His-His-Thr-Thr, chitin binding domain, and the like.

A fusion protein may be a means by which the
25 recombinant antigen protein can be easily detected. For example, the fusion component can itself be a detectable moiety, such as fluorescent protein (fluorescent green protein, fluorescent yellow protein, and the like), or alternatively can be one member of a specific binding

pair (such as biotin and streptavidin, for example) which can be detected by reacting with the other member conjugated to a detectable substance.

The foregoing elements can be combined to produce
5 vectors suitable for use in the methods of the invention. Those of skill in the art would be able to select and combine the elements suitable for use in their particular system.

The introduced nucleic acid molecule can be
10 incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient
15 cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of
20 different species.

Suitable prokaryotic vectors include plasmids such as those capable of replication in E. coli (for example, pBR322, ColE1, pSC101, PACYC 184, itVX, pRSET, pBAD (Invitrogen, Carlsbad, CA), and the like). Such plasmids
25 are disclosed by Sambrook (cf. "Molecular Cloning: A Laboratory Manual", second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)). Bacillus plasmids include pC194, pC221, pT127, and the like, and are disclosed by Gryczan (In: The

- Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include plJ101 (Kendall et al., J. Bacteriol. 169:4177-4183, 1987), and streptomyces bacteriophages such as ϕ C31 (Chater et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kiado, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John et al. (Rev. Infect. Dis. 8:693-704, 1986), and Izaki (Jpn. J. Bacteriol. 33:729-742, 1978).
- 10 Suitable eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, pCDN3.1 (Invitrogen), and the like, or their derivatives. Such plasmids are well known in the art (Botstein et al., Miami Wntr. Symp. 19:265-274, 1982); Broach, In: "The
- 15 Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, Cell 28:203-204, 1982); B(Dilon et at., J. Clin. Hematol. Oncol. 10:39-48, 1980); Maniatis, In: Cell Biology: A
- 20 Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608 (1980).

Once antibody:antigen complexes have been formed and unbound antigen washed away under suitable conditions, the antibody:antigen complexes can be detected using one

25 of several techniques known in the art. Suitable washing conditions are known to those skilled in the art (see, for example, Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995). Exemplary washing conditions are shown in the examples below.

For detection in the case of recombinant antigens, expression vectors can be used that form chimeric fusion peptides as described above. The epitope tagged antigen can be detected using an antibody specific for the tag
5 sequence. This antibody may be itself detectably labeled or can be detected with a third detectably-labeled antibody. Alternatively, the antigen can be complexed with biotin and detected using detectably-labeled avidin or streptavidin. The antigen itself can also be
10 detectably labeled, such as with a fluorescent dye compound.

The term "detectably labeled" as used herein is intended to encompass antigen directly coupled to a detectable substance, such as a fluorescent dye, and
15 antigen coupled to a member of binding pair, such as biotin/streptavidin, or an epitope tag that can specifically interact with a molecule that can be detected, such as by producing a colored substrate or fluorescence.

20 Substances suitable for detectably labeling proteins include fluorescent dyes such as fluorescein isothiocyanate (FITC), fluorescein, rhodamine, tetramethyl-rhodamine-5-(and 6)-isothiocyanate (TRITC), Texas red, cyanine dyes (Cy3 and Cy5, for example), and
25 the like; and enzymes that react with colorimetric substrates such as horseradish peroxidase. The use of fluorescent dyes is generally preferred in the practice of the invention as they can be detected at very low amounts. Furthermore, in the case where multiple

antigens are reacted with a single array, each antigen can be labeled with a distinct fluorescent compound for simultaneous detection. Labeled spots on the array are detected using a fluorimeter, the presence of a signal
5 indicating an antigen bound to a specific antibody.

The formation of antibody:antigen complexes can be performed under a variety of conditions to identify antibodies with varying binding characteristics. Antigen-containing reaction solutions can contain varying
10 degrees of salt or be conducted at varying pH levels. In addition, the binding reaction can be carried out at varying temperatures. Each set of conditions will identify antibodies with different affinity for the antigen. For example, antibodies that bind at pH 2 may
15 have utility under highly acidic conditions such as those that exist in the stomach. Similarly, antibodies that bind at temperatures near boiling may be useful in studying thermophilic organisms. In general pH conditions will range from 2 - 10 (most preferably around
20 pH 8), temperatures from 0° C - 100°C and salt conditions from 1 μ M to 5 M (in the case of NaCl).

Affinity constants are a measure of the interaction between a particular ligand and its cognate receptor. The "binding affinity" or the measure of the strength of
25 association between a particular antibody:antigen interaction is generally measured by affinity constants for the equilibrium concentrations of associated and dissociated configurations of the antibody and its antigen. Preferably the binding of the antigen should

occur at an affinity of about $k_a = 10^{-6}M$ or greater to be useful for the present invention, with greater than about $10^{-7}M$ being more preferable, and most preferably between about $10^{-8}M$ and about $10^{-11}M$. Antibody fragments will
5 generally have affinities in the range of about $10^{-6}M$ to $10^{-7}M$.

In another embodiment of the invention, microarrays of uncharacterized antibodies are used to compare the protein expression profiles of cells. For example,
10 comparisons can be made between a population of cells from one tissue, such as arterial endothelial cells, and a second tissue, such as venous endothelial cells or from cells derived from a particular tissue but from different species. Comparisons can be made between normal cells
15 and cells from the same tissue type that originate from an individual with a pathogenic disorder. For example, comparisons can be made between normal cells and cancer cells. Comparisons can additionally be made between cells in a resting state and cells in an activated state,
20 for example, resting T-cells and activated T-cells.

In another example, the disclosed arrays are useful for evaluating the expression of proteins by pathogens, such as, for example, bacteria, parasites, viruses, and the like. A solution (such as a lysate) made from the
25 pathogen which represents all proteins expressed by the pathogen can be used to contact an antibody array to identify antibodies recognizing pathogen-expressed proteins. These antibodies have utility as diagnostic agents as well as potential therapeutics.

Cellular lysates can be used as "antigens" as described above and reacted with two identical microarrays. Antibodies reactive in one array but not the other would indicate the presence of a differentially
5 expressed protein. This antibody is then useful for the subsequent isolation and identification of those proteins that are different in two populations of cells. In the case of normal and cancer cells, for example, one may be able to identify proteins expressed in the cancer cell
10 that contribute to its malignant state.

In a further aspect of the invention, microarrays can be composed of previously characterized antibodies. These microarrays have a variety of uses, one of which is cell profiling. For example, an array can be composed of
15 antibodies that recognize a set of antigens known to be present in activated T-cells but not in resting T-cells. A population of T-cells can then be lysed and the lysate contacted with the array to determine if the population has the profile of activated or resting T-cells.

20 Microarrays and the methods disclosed herein can be used in methods of diagnosing particular disorders. For example, a collection of antibodies specific for a range of antigens associated with one or more disorders can be arrayed and contacted with a bodily fluid containing
25 antigens whose presence, or absence, would indicate a particular disorder. The advantage of using a microarray over a conventional immunoassay is the ability to include a population of antibodies diagnostic for a variety of

disorders on a single surface, significantly reducing time, costs and materials needed to effect a diagnosis.

For example, if a patient presents with symptoms that are characteristic of several distinct disorders which can be distinguished on the basis of the presence or absence of one or more proteins, a single microarray assay could be used to make a specific diagnosis, thus allowing the patient to be properly treated. Patients suffering from stroke or brain infarcts release several proteins into cerebrospinal fluid; examples of which are neuron specific enolase (NSE) from neuronal cells and S-100 from glial cells and astrocytes. Such proteins are not released in conditions that may have similar symptoms, such as drug reactions, making proper diagnosis more difficult. A diagnostic array could readily detect these and other proteins in the CSF, leading to a rapid clinical diagnosis and treatment.

In another aspect of the invention microarrays are employed to characterize protein expression patterns using nucleic acid samples. Briefly, nucleic acid molecules from a whole cell or tissue are applied to a solid support using a microarray format. The arrayed nucleic acid samples are then contacted with a nucleic acid probe specific for a gene encoding a known protein. The probe solution is left in contact with the array for an amount of time sufficient to allow sample:probe complexes to form, then the unbound probe is washed away under suitable conditions (see, for example, Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995

WO 99/40434

32

PCT/US99/02442

and the examples below). Bound probe is detected at one or more nucleic acid sample spots using one of a variety of detection methods.

This aspect of the invention has a variety of uses. For example, the microarray can be constructed from nucleic acid samples isolated from a single tissue type but from a large number of species, with each spot representing a particular species. Thus in a single assay format one can determine the evolutionary development of the protein represented by the probe. Similarly, the microarray can be constructed of multiple tissue types from a single species, or from different developmental stages of a single species (or multiple species) thus simply and efficiently determining tissue expression of the protein represented by the probe. For example, a microarray can be constructed with arrayed samples representing all the developmental stages of *Drosophila*, a well known organism the study of which has led to a greater understanding of mammalian physiology and development.

The nucleic acid sample can be messenger ribonucleic acid (mRNA) or can be complementary dexoyribonucleic acid (cDNA), including EST fragments. Methods for extracting and isolating nucleic acids from cells are well known in the art (for example phenol extraction/ethanol precipitation, ammonium acetate precipitation, cesium chloride gradients, and the like), as are methods for generating cDNA (see, for example, "Molecular Cloning: A Laboratory Manual," second edition, edited by Sambrook,

WO 99/40434

33

PCT/US99/02442

Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989; and Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995, both of which are incorporated by reference herein). Microarrays of these nucleic acids are created
5 using the methods described above. Techniques for coupling nucleic acids to solid supports used to construct microarrays are well known in the art, including the poly-L-lysine and phenylboronic acid methods described in the Examples below.

10 The nucleic acid probes used in the invention methods can be designed based on the sequence of a gene encoding a known protein or can be an EST fragment, as described above. One skilled in the art can readily design such probes based on the known sequence using
15 methods of computer alignment and sequence analysis known in the art (e.g. "Molecular Cloning: A Laboratory Manual", second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989; Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995). The probe can comprise any number of nucleotides
20 but will preferably be not fewer than 10 nucleotides and preferably not more than about 300 nucleotides in length.

The probes of the invention can be labeled by standard labeling techniques such as with a radiolabel,
25 enzyme label, fluorescent label, biotin-avidin label, chemiluminescent label, and the like. After hybridization, the probes may be detected using known methods. Preferred labels are fluorescent labels, as described above.

The nucleic acid probes of the present invention include RNA as well as DNA probes and nucleic acids modified in the sugar, phosphate or even the base portion as long as the probe still retains the ability to specifically hybridize under conditions as disclosed herein. Such probes are generated using techniques known in the art.

The term "hybridize" as used herein refers to a method of interacting a nucleic acid sequence with a DNA or RNA molecule in solution or on a solid support, such as cellulose or nitrocellulose. If a nucleic acid sequence binds to the DNA or RNA molecule with sufficiently high affinity, it is said to "hybridize" to the DNA or RNA molecule. The strength of the interaction between the probing sequence and its target can be assessed by varying the stringency of the hybridization conditions. Various low to high stringency hybridization conditions may be used depending upon the specificity and selectivity desired. Stringency is controlled by varying salt or denaturant concentrations. Examples of hybridization conditions are shown in the Examples below. Those skilled in the art readily recognize how such conditions can be varied to vary specificity and selectivity. For example, under highly stringent hybridization conditions only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having even one or two mismatches out of 20 contiguous nucleotides.

In a further aspect of the invention, microarrays can be composed of randomly generated polynucleotides (DNA or RNA) and contacted with proteins to identify unique binding pairs. Polynucleotides are now known to
5 bind to proteins and may have potential as diagnostics and therapeutics (see, for example, Allen, et al, Virology 209(2):327-336, 1995; Binkley, et al, Nucleic Acids Res. 23(16):3198-3205, 1995). Polynucleotides can be evaluated in very large numbers using the methods
10 disclosed herein thus increasing the likelihood of identifying a useful binder.

The invention will now be described in greater detail by reference to the following non-limiting examples.

15

Examples

Example I - Nucleic Acid Microarrays

The following procedures are conducted at room temperature and using double distilled water unless otherwise noted. These methods are applicable to arrays
20 of polypeptides or polynucleic acids.

Glass slides are prepared as follows: NaOH (50 g) is dissolved in 150 ml of double distilled water (ddH₂O), then 200 ml of 95% EtOH is added while stirring. If the solution becomes cloudy, ddH₂O is added until it becomes
25 clear. Approximately 30 glass slides (Gold Seal, Cat. No. 3010) are soaked in the NaOH/EtOH solution for 2 hours, shaking. The slides are then rinsed three times with ddH₂O. The slides are next soaked in a poly-L-lysine

WO 99/40434

36

PCT/US99/02442

solution (70 ml poly-L-lysine (Sigma Cat. No. 8920) to 280 ddH₂O) for 1 hour. Excess liquid is removed by spinning the slides in a rack on a microtiter plate carrier at 500 rpm. The slides are dried at 40° C for 5 minutes, then stored in a closed box for at least 2 weeks prior to use.

A cDNA microarray is prepared as follows: Total mRNA is isolated from tissue (for example, nerve cells) of a variety of species representative of different classes of organisms such as *Drosophila*, nematode, salmon, clam, chicken, mouse, dog, goat, spider monkey, chimpanzee, human, and the like, by the FastTrac method (Stratagene, La Jolla, CA) or other common methods. mRNA is also obtained from a variety of unicellular organisms such as *E. coli*, yeast, *B. subtilis*, mycoplasma and the like. Eukaryotic mRNA is enriched from total RNA using oligo(dT) cellulose (Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed. 1995, pgs 4-11 - 4-12). Equivalent amounts (for example, 1 µg) of mRNA from each source are placed in a separate well of one or more 96 well microtiter plates and precipitated with cold EtOH. The precipitate is rinsed with 70% EtOH and allowed to dry.

The dried mRNA is resuspended in 3x SSC (sodium chloride/sodium citrate - 20X solution is 3 M NaCl (175g/L) 0.3 M trisodium citrate 2H₂O (88g/L adjusted to pH 7.0 with 1 M HCl) then spotted onto a previously prepared glass slide using an array device (for example, Shalon and Brown (WO 95/35505, published 12/28/95)). The

WO 99/40434

37

PCT/US99/02442

prepared array can be kept for a long period of time before probing, however, if the slides are to be kept for long periods of time, stability is increased by converting each mRNA sample into cDNA using techniques known in the art, such as PCR.

The array is rehydrated by suspending the slide over a dish of ddH₂O (50°C) for approximately one minute. The slide is quickly (approximately 3 seconds) dried by placing it on a surface heated to 100° C (mRNA side up). The mRNA is crosslinked to the poly-L-lysine coating of the slide using ultraviolet radiation using a Stratalinker™ UV device according to the manufacturer's instructions (Stratagene) set at 60 milliJoules.

The slides are next soaked in a solution of 5 grams of succinic anhydride (Aldrich Cat. No. 23,969-0) dissolved in 315 ml of N-methyl-pyrrolidinone (Aldrich Cat. No. 32,863-4) plus 35 mls of 0.2 M sodium borate (brought to pH 8.0 with NaOH) for 15 minutes with shaking. The slide is then transferred to a 95° C water bath for 2 minutes followed by 95% EtOH for 1 minute. Excess liquid is removed from the slides by spinning a rack of slides on a microtiter plate carrier at 500 rpm.

A probe sequence of a known protein (for example, human nerve growth factor, GeneBank Accession No. E03589) is labeled using standard protocols, for example by using a CyDye Nick Translation kit (Amersham). The labeled probe (approximately 1 µg/ml) is resuspended in 4X SSC (10 µl) to which is added 0.2 µl 10% sodium dodecyl sulfate (SDS). The probe is boiled for 2 minutes, then

WO 99/40434

38

PCT/US99/02442

cooled for 10 seconds and transferred to the array by pipette. The array is covered by a 22mm x 22mm cover slip, and the slide is placed in a humid hybridization chamber and submerged into a hot water bath ($\geq 75^{\circ}\text{C}$).

- 5 The slide is left in the bath for 10 - 24 hours, then the cover slip is removed and the slide rinsed in 0.2X SSC with 0.1% SDS several times. Excess wash buffer is removed by centrifugation on a microtiter plate carrier as described above. The slide is scanned using a
- 10 spectrofluorometer, such as the ScanArray 3000 (General Scanning Inc., Watertown, MA). For probes labeled with Cy5, for example, fluorescence is measured at 670 nm.

- Localization of spots on the array to which the probe hybridizes indicates that the species represented
- 15 by the spot expresses a protein similar or identical to the probe protein.

- The procedure outlined below is an alternative method for binding arrayed molecules to a solid support, using an SA(OCH₂CN)-X-NHS linkage (see, for example, US
- 20 5,594,111, issued 1/14/97; 5,648,470, issued 5/15/97; US 5,623,055, issued 4/22/97; all of which are incorporated by reference herein).

- Glass slides (Fisher Catalog No. 12-544-4) are soaked in an acid bath (1 hour in 0.1 M HCl), then washed
- 25 with water and dried at room temperature. The slides should not be aggressively dried, such as in an oven. The slides are next soaked in a silane solution overnight at room temperature (5% APTES (3-aminopropyl-

WO 99/40434

39

PCT/US99/02442

triethoxysilane, Aldrich 28,177-8), 0.3% DIEA (Sigma) v/v in EtOH). The slides may be sonicated for 10 - 15 minutes right after being placed in the APTES solution.

5 The slides are rinsed with isopropyl alcohol, then sonicated in isopropyl alcohol for several minutes. Sonication should remove any white silane residue on the slides. If the residue remains, the slides should be discarded. After sonication, the slides are left to cure/dry for at least 24 hours before use.

10 The cured slides are next soaked in a linker solution overnight at room temperature. The linker solution is made by dissolving 115 mg of 9Y SA(OCH₂CN)-X-COOH (Prolix, Bothell, WA) in 1 ml dimethylformamide (DMF) plus 60 µl DIEA, then adding 60 mg TSTU (Sigma) and
15 leaving for 15 minutes at room temperature. This stock is diluted in 270 ml of isopropyl alcohol plus 270 µl DIEA before using.

The slides are removed from the linker solution and soaked in 1 M NH₂OH, 1mM EDTA, 0.1 M NaHCO₃ (pH 10) for 4
20 hours at room temperature. This solution is removed, the slides are extensively washed with water then let air dry at room temperature. The slides can be stored at room temperature away from light before using to make arrays.

25 Example II - Determination of Optimal Concentrations of Antibody and Antigen

Various concentrations (1 µg/µl, 100 ng/µl, 10 ng/µl, 1 ng/µl) of total mouse IgG or a mouse monoclonal anti-

WO 99/40434

40

PCT/US99/02442

PLC-gamma were spotted on aldehyde slides (Cel Associates, Inc., Houston, Texas), which allow non-covalent attachment of proteins. Using a manual 8 pin hand arrayer the slides were blocked for 1 hour with PBST (phosphate buffered saline and 0.10% Tween 20), and 3% milk protein. The slides were subsequently washed three times, 15 minutes each, in PBST. Duplicate slides were incubated with 50 μ l of goat anti-mouse IgG antibody (GAMG) conjugated with CY3 or CY5 fluorescent dye compounds (Amershan, Arlington Heights, Illinois) at 10 μ g/ml or 1 μ g/ml. Slides were then washed for 15 minutes in PBST three additional times and dried by centrifugation prior to scanning. Binding was detected as shown in Table 1 below.

WO 99/40434

41

PCT/US99/02442

Table 1

Antibody	Conc.	Antigen	Conc.	Detection Level
PLC-gamma	1 µg/µl	GAMG-CY3	10 µg/ml	+++
	100 ng/µl		10 µg/ml	+++
	10 ng/µl		10 µg/ml	+
	1 ng/µl		10 µg/ml	-
mouse IgG	1 µg/µl	GAMG-CY3	10 µg/ml	+++
	100 ng/µl		10 µg/ml	+++
	10 ng/µl		10 µg/ml	+
	1 ng/µl		10 µg/ml	-
PLC-gamma	1 µg/µl	GAMG-CY3	1 µg/ml	+
	100 ng/µl		1 µg/ml	+
	10 ng/µl		1 µg/ml	-
	1 ng/µl		1 µg/ml	-
mouse IgG	1 µg/µl	GAMG-CY3	1 µg/ml	+
	100 ng/µl		1 µg/ml	+
	10 ng/µl		1 µg/ml	-
	1 ng/µl		1 µg/ml	-
PLC-gamma	1 µg/µl	GAMG-CY5	10 µg/ml	+++
	100 ng/µl		10 µg/ml	+++
	10 ng/µl		10 µg/ml	+
	1 ng/µl		10 µg/ml	-
mouse IgG	1 µg/µl	GAMG-CY5	10 µg/ml	+++
	100 ng/µl		10 µg/ml	+++
	10 ng/µl		10 µg/ml	+
	1 ng/µl		10 µg/ml	-
PLC-gamma	1 µg/µl	GAMG-CY5	1 µg/ml	+
	100 ng/µl		1 µg/ml	+
	10 ng/µl		1 µg/ml	-
	1 ng/µl		1 µg/ml	-
mouse IgG	1 µg/µl	GAMG-CY5	1 µg/ml	+
	100 ng/µl		1 µg/ml	+
	10 ng/µl		1 µg/ml	-
	1 ng/µl		1 µg/ml	-

+++ strong signal, ++ moderate signal

+ weak signal, - no signal

WO 99/40434

42

PCT/US99/02442

Example III - Comparison of Solid Supports

Serial dilutions (1 µg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml) of mouse IgG or PLC-gamma were hand arrayed onto aldehyde, polystyrene, nitrocellulose and Surmodics slides. Aldehyde, nitrocellulose, polystyrene and Surmodics slides were purchased from various outside vendors (aldehyde Slides - Cel Associates, Inc., Houston, Texas; nitrocellulose Slides - Molecular Probes, Inc., Eugene, Oregon; polystyrene Slides - Nunc, Inc., Naperville, Illinois; Surmodics Slides - Surmodics, Inc., Eden Prairie, Minnesota). Surmodics slides have an undisclosed polymer on the glass surface which forms a covalent linkage with proteins under the appropriate conditions (described by the manufacturer).

Following hand arraying of the antibodies (approximately 20-30 nanoliters per spot), the nitrocellulose, aldehyde, and polystyrene slides were immediately blocked for 1 hour with PBST and 3% milk, washed 3 times with PBST, and hybridized with 50 µl of GAMG-CY3 for 30 minutes. Surmodics slides were incubated overnight in a moist salt chamber as recommended by the manufacturer. The following day, the Surmodics slides were processed as described above. Following hybridization all of the various slides were washed 3 times in PBST, dried and scanned using a Scan Array 3000 fluorescent scanner.

All of the slides tested allowed for the detection of antigen:antibody binding at higher concentrations of antibody. The Aldehyde and Nitrocellulose treated slides

WO 99/40434

43

PCT/US99/02442

were the most efficient at binding antibody, and antibody:antigen interaction could be detected at 1ng/ μ l.

Example IV - Detection of Binding Using Non-Fluorescent
5 Methods

Positively charged nylon filters (Zeta Probe Membranes, Bio Rad Laboratories, Hercules, CA) were hand arrayed using 1 μ l of anti-His, anti-V5, anti-thioredoxin (anti-Thio), anti-FOS, anti-PLC-gamma and anti-CREB antibodies
10 (Invitrogen, Carlsbad, CA; all antibodies were approximately 1 mg/ml). Filters were blocked for 1 hour with PBST and 3% milk, washed three times with PBST, and incubated with 1 μ g/ml biotinylated D1 protein for three hours at room temperature. D1 is a creatine kinase
15 fusion protein isolated from a human fetal heart cDNA library and cloned into the pBAD-Thio-His-TOPO vector (Invitrogen, Carlsbad, CA) to create a Thioredoxin-V5-His-creatine kinase fusion protein. D1 was biotinylated using the EZ-Link™ Sulfo-NHS-LC Biotinylation Kit
20 (Pierce, Rockford, IL) used according to the manufacturer's instructions).

Following three additional washes with the same buffer, filters were treated with streptavidin/alkaline phosphatase conjugate or streptavidin/horseradish
25 peroxidase conjugate (Boehringer Mannheim, GmbH Germany) for 1 hour at room temperature.

The filters were washed 5 times with PBST, dried, and developed by immersion in ECL chemiluminescent substrate (ECL - Amersham, Arlington Heights, Illinois)

WO 99/40434

44

PCT/US99/02442

or the chromogenic substrate BCIP/NBT (Sigma Chemicals, St. Louis, MO). Filters developed with ECL were exposed to Kodak chemiluminescent film for 1 to 10 seconds.

5 The results are shown in Figures 1A, 1B, and 1C. In all cases, only the antibodies specific for epitopes on the fusion protein antigen were detectable, and only in the arrayed spots, showing that the system has both good signal to noise ratio and specificity.

10 The experiment was repeated using an array created with an automated arrayer. Antibodies (1 mg/ml) were spotted using an automated 96 pin microarrayer developed at Invitrogen. Fifteen negative control antibodies (assorted mouse monoclonals) were arrayed along with the three positive control antibodies (anti-His, anti-Thio, 15 anti-V5). Filters were treated as described above using the alkaline phosphatase conjugate and the chromogenic substrate BCIP/NBT.

As can be seen in Figure 2, binding and detection of antibody:antigen complexes was highly specific and 20 sensitive.

Example V - Evaluation of Antibody Affinity

25 Anti-His, anti-V5, anti-FOS, anti-PLC-gamma, 25C1DG, and anti-VEGF (vascular endothelial growth factor) antibodies were arrayed on a nitrocellulose slide and reacted with biotinylated D1 protein as previously described. Binding was detected with streptavidin - Cy3 as described above. The anti-V5 antibodies spots showed red, the anti-His spots showed green, while the negative

WO 99/40434

45

PCT/US99/02442

controls were undetectable (see Figure 3). When viewed in a black and white drawing, relative increase in binding affinity is visualized by an increase of white in a given area. The color of the spots generally indicates a higher amount of fluorescently labeled antigen present, and thus indicates relative binding affinity between antibody and antigen. Colors, in descending order from highest to lowest affinity, are white, red, yellow, green, and blue. Using this technique, multiple antibodies can be tested for their affinity to a single antigen.

Example VI - Polyclonal Antibody Microarrays

To demonstrate specific binding to polyclonal antibodies, six antibodies were arrayed by hand on a nitrocellulose slide, three polyclonal antibodies (anti-E12 (unpurified rabbit polyclonal sera to a His-V5-thioredoxin-thymidine kinase fusion protein), anti-lexA (lexA repressor protein), and anti-GFP (Green fluorescent protein)) and three monoclonal antibodies (anti-V5, anti-His and anti-GalU (a mammalian transcription factor)). The slide was blocked with PBST and 3% milk for 1 hour at room temperature, and incubated with the E12-biotin conjugate, prepared according to the protocol used for D1 protein. Following extensive washing with PBST, the slides were incubated with streptavidin-CY3 conjugate (Amersham, Arlington Heights, IL) for 1 hour at room temperature, washed 5 times with PBST and dried by centrifugation prior to scanning on the Scan Array 3000.

WO 99/40434

46

PCT/US99/02442

As can be seen in Figure 4, binding was detected with both the antigen specific polyclonal antibody (anti-E12) and the antigen specific monoclonal antibodies (anti-His, anti-V5) and not with any of the negative control antibodies.

Example VII - Microarray Analysis of Labeled Cell Lysate

A series of experiments were conducted to determine if a microarray of antibodies could specifically detect antigens in a cell lysate.

CHO cells expressing high levels of beta-galactosidase were grown to confluency in a T-175 flask. (Hams media with Pen/Strep, and L-glutamine plus 10% FCS, at 37°C with 5% CO₂) Cells were harvested using Trypsin/EDTA. NP40 extracts were prepared by pelleting the cells (10⁷ cells), washing once in PBS and resuspending in 5% NP40. Cell debris was removed by centrifugation. Soluble protein was biotinylated using a Pierce biotinylation kit according to the manufacturer's instructions.

Nitrocellulose slides (see above) containing arrayed monoclonal antibodies (anti-beta-gal, anti-His, anti-Thio, anti-V5, anti-FOS, anti-PLC-gamma, anti-VEGF and 25C10G (an anti-CREB antibody) were blocked, washed, hybridized and developed with streptavidin-CY3 as described in Example VI *supra*. As can be seen in Figure 5A, beta-galactosidase binding was seen, however, some non-specific binding was detected as well.

WO 99/40434

47

PCT/US99/02442

The experiment was repeated, except that after centrifugation of the extract, soluble protein was dialyzed overnight against 50 mM phosphate buffer at 4°C prior to biotinylation. As can be seen in Figure 5B, much of the non-specific binding seen in the previous experiment was eliminated.

In the next experiment dialyzed extract containing the biotinylated soluble proteins was adjusted to 10% glycerol to reduce non-specific hydrophobic interactions. Furthermore, the sodium chloride concentration was adjusted to 0.2 M NaCl to increase specific ionic interactions. All other conditions remained identical. As can be seen in Figure 5C, all non-specific binding was eliminated using this protocol.

15

While the foregoing has been presented with reference to particular embodiments of the invention, it will be appreciated by those skilled in the art that changes in these embodiments may be made without departing from the principles and spirit of the invention, the scope of which is defined by the appended claims.

20

WO 99/40434

48

PCT/US99/02442

That which is claimed is:

1. A method of identifying antibodies having binding affinity for an antigen, said method comprising:
 - (a) contacting an array of uncharacterized
5 antibodies on a solid surface with at least one antigen;
and
 - (b) identifying the antibodies to which the antigen binds.
- 10 2. A method according to claim 1 wherein the antigen is a protein.
3. A method according to claim 1 wherein the antigen is an intact cell.
4. A method according to claim 1 wherein the antigen is a cell lysate.
- 15 5. A method according to claim 2 wherein the protein is recombinant.
6. A method according to claim 5 wherein the protein is full-length.
7. A method according to claim 5 wherein the
20 protein is a protein fragment.
8. A method according to claim 7 wherein the protein fragment is encoded by an EST fragment.
9. A method according to claim 1 wherein the antibodies are monoclonal antibodies.
- 25 10. A method according to claim 1 wherein the antibodies are polyclonal antibodies.
11. A method according to claim 1 wherein the antibodies are antibody fragments.

WO 99/40434

49

PCT/US99/02442

12. A method according to claim 11 wherein the antibody fragments are single chain antibodies.

13. A method according to claim 1 wherein the antibodies are recombinant antibodies.

5 14. A method according to claim 1 wherein the antigen is detectably labeled.

15. A method according to claim 14 wherein the detectable label is a fluorescent moiety, avidin, streptavidin, or biotin.

10 16. A method according to claim 1 wherein the antigen is a fusion protein comprised of an epitope tag or a fluorescent protein.

15 17. A method according to claim 1 wherein the binding affinity of said antibody for said antigen is determined by iterative washing of said solid surface with a suitable diluent and detecting when antigen is no longer released therefrom.

18. A method of comparing protein expression in two or more populations of cells, said method comprising:

20 (a) contacting an array of antibodies on a solid surface with a cell lysate of a first cell population, generating a first binding pattern;

(b) contacting a duplicate array of antibodies on a solid surface with a cell lysate of a second cell
25 population, generating a second binding pattern; and

(c) comparing the binding pattern of the first cell lysate with the binding pattern of the second cell lysate.

30 19. A method according to claim 18 wherein the antibodies are uncharacterized antibodies.

WO 99/40434

50

PCT/US99/02442

20. A method according to claim 18 wherein the antibodies are recombinant antibodies.

21. A method according to claim 18 wherein the first cell lysate is derived from normal cells and the
5 second cell lysate is derived from abnormal cells.

22. A method according to claim 21 wherein the abnormal cells are cancer cells.

23. A method according to claim 18 wherein the first cell lysate is derived from normal cells in a
10 resting state and the second cell lysate is derived from normal cells in a stimulated state.

24. A method according to claim 18 wherein the difference between the first and second set of cells is the presence of a different detectable label.

15 25. A method for determining the effect of varying binding conditions on the binding affinity of antibodies to a specific antigen, said method comprising:

(a) contacting an array of antibodies on a solid surface with at least one antigen under a first set of
20 binding conditions, generating a first binding pattern;

(b) contacting a duplicate array with the antigen under a second set of binding conditions, generating a second binding pattern;

(c) comparing the first and second binding
25 patterns.

26. A method according to claim 21 wherein said varying binding conditions comprise varying pH, temperature, salt concentration, and/or duration.

WO 99/40434

51

PCT/US99/02442

27. A method for characterizing a cell, based on the pattern of protein expression produced thereby, said method comprising:

- 5 (a) contacting an array of antibodies on a solid surface with a cell lysate; and
- (b) identifying the profile of antibodies to which components of the lysate binds.

28. A method of diagnosing a disorder, said method
10 comprising:

- (a) contacting an array of antibodies specific for one or more antigens characteristic of a disorder with a biological sample obtained from a subject under conditions suitable for the formation of an
15 antigen:antibody complex, wherein the presence of the antigens in the biological sample would be indicative of the disorder; and
- (b) detecting the formation of any antibody:antigen complexes.

20 29. A method according to claim 28 wherein the biological sample is cerebral spinal fluid, blood, plasma, urine, feces, saliva, tears, or extracted tissue.

30. A method according to claim 29 wherein the disorder is stroke, cerebral hemorrhage, myocardial
25 infarction, peripheral blood clots, diabetes, cancer, Alzheimer's disease, and sepsis.

31. A kit comprising:

- (a) an array of uncharacterized antibodies on a solid surface; and
- 30 (b) instructions for using the array.

WO 99/40434

52

PCT/US99/02442

32. A kit according to claim 31 wherein the instructions are for identifying antibodies to a specific antigen, comparing protein expression in two or more populations of cells, characterizing a cell based on the pattern of protein expression produced thereby, or determining the effect of varying binding conditions on the binding affinity of the antibodies.

33. A kit according to claim 31 wherein the antibodies are monoclonal antibodies, polyclonal antibodies or antibody fragments.

34. A kit according to claim 33 wherein the antibody fragments are single chain antibodies.

35. A kit according to claim 31 wherein the antibodies are recombinant antibodies.

36. A kit according to claim 31 further comprising reagents for detecting an antigen and instructions for use thereof.

37. A kit comprising:

(a) an array of antibodies on a solid surface; and
(b) instructions for using the array; wherein the instructions are for diagnosing a disorder, characterizing a cell based on the pattern of protein expression produced thereby, or comparing protein expression in two or more populations of cells.

38. A kit according to claim 37 further comprising reagents for detecting an antigen and instructions for use thereof.

39. A kit according to claim 37 wherein the antibodies are recombinant antibodies.

WO 99/40434

53

PCT/US99/02442

40. A kit according to claim 37 wherein the antibodies are single chain antibodies.

41. A method of comparing protein expression patterns, said method comprising:

5 (a) contacting a microarray of nucleic acid samples derived from different sources with one or more nucleic acid probes and

(b) identifying the sample or samples to which the probe(s) binds.

10 42. A method according to claim 41 wherein the microarray comprises nucleic acid samples derived from a single tissue type but from different species.

15 43. A method according to claim 41 wherein the microarray comprises nucleic acid samples derived from a single species but from different tissue types.

44. A method according to claim 41 wherein the microarray comprises nucleic acid samples derived from the same tissue type at different developmental stages.

20 45. A method according to claim 41 wherein the nucleic acid samples are comprised of mRNA or cDNA.

46. A method according to claim 41 wherein the probe is detectably labeled.

47. A method according to claim 46 wherein the detectable label is a fluorescent label.

25 48. A method according to claim 18 wherein the first and second cell lysates are derived from cells from a single tissue type but from different species.

WO 99/40434

54

PCT/US99/02442

49. A method according to claim 18 wherein the first and second cell lysates are derived from cells from a single species but from different tissue types.

50. A method according to claim 18 wherein the
5 first and second cell lysates are derived from cells from the same tissue type at different developmental stages.

WO 99/40434

PCT/US99/02442

2/7

FIG. 2

• •
• •

• •
• •

• •
• •

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02442

A. CLASSIFICATION OF SUBJECT MATTER																										
IPC(6) : G01N 33/53, 33/48; 33/543 US CL : 435/7.1, 6; 436/518, 536, 501, 543 According to International Patent Classification (IPC) or to both national classification and IPC																										
B. FIELDS SEARCHED																										
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.1, 6; 436/518, 536, 501, 543																										
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																										
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CAS Search terms: antibodies, antigens, microarrays, nucleic acids, probes, libraries, solid support																										
C. DOCUMENTS CONSIDERED TO BE RELEVANT																										
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																								
X	US 4,591,570 A (CHANG) 27 May 1986, col. 1, lines 47-68; col. 2, lines 44-58; col. 3; lines 30-38; col. 4, lines 1-66; col. 5, Examples up to col. 8, line 14.	1,4,6,9,10, 17 ----- 5, 7-8, 11-16																								
X	RAMSAY, G. DNA chips: State-of-the art, Nature Biotechnology January 1988. Vol. 16, No. 1, pages 40-44, especially pages 41-43.	41-50																								
Y	ZIEGER, A. et al. Cucumber Mosaic Cucumovirus Antibodies from a Synthetic Phage Display Library. Virology. 1995, Vol. 214, pages 235-238, especially page 235.	1-2, 6-7, 9-13																								
Y	US 4,483,929 A (SZOKA) 20 November 1984, col. 8, line 38-47; col. 10, lines 48-61.	9-11																								
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																										
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>* T</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>* A</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td></td> </tr> <tr> <td>* B</td> <td>earlier document published on or after the international filing date</td> <td>* X</td> </tr> <tr> <td>* L</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>* O</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>* Y</td> </tr> <tr> <td>* P</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td></td> <td></td> <td>* E</td> </tr> <tr> <td></td> <td></td> <td>document member of the same patent family</td> </tr> </table>			* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	* A	document defining the general state of the art which is not considered to be of particular relevance		* B	earlier document published on or after the international filing date	* X	* L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	* O	document referring to an oral disclosure, use, exhibition or other means	* Y	* P	document published prior to the international filing date but later than the priority date claimed	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art			* E			document member of the same patent family
* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																								
* A	document defining the general state of the art which is not considered to be of particular relevance																									
* B	earlier document published on or after the international filing date	* X																								
* L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																								
* O	document referring to an oral disclosure, use, exhibition or other means	* Y																								
* P	document published prior to the international filing date but later than the priority date claimed	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																								
		* E																								
		document member of the same patent family																								
Date of the actual completion of the international search 25 MARCH 1999		Date of mailing of the international search report 28 APR 1999																								
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer: T. WESSENDORF Telephone No. (703) 308-0196																								

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02442

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EKINS, R. et al. Multianalyte microspot immunoassay. The microanalytical 'compact disk' of the future. Ann. Biol. Clin. 1992, Vol. 50, pages 337-353, especially page 337.	1
A	MARSHALL, A. et al. DNA chips: An array of possibilities. Nature Biotechnology. January 1998, Vol. 16, No. 1, pages 27-31.	1-50